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# EPR characterisation of the triplet state in photosystem II reaction centers with singly reduced primary acceptor $Q_A$

W. Onno Feikema[a], Peter Gast[a,*], Irina B. Klenina[b], Ivan I. Proskuryakov[b]

[a]*Department of Biophysics, Huygens Laboratory, P.O. Box 9504, 2300 RA Leiden, The Netherlands*
[b]*Institute of Basic Biological Problems RAS, Pushchino 142290, Russia*

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**Abstract**

The triplet states of photosystem II core particles from spinach were studied using time-resolved cw EPR technique at different reduction states of the iron–quinone complex of the reaction center primary electron acceptor. With doubly reduced primary acceptor, the well-known photosystem II triplet state characterised by zero-field splitting parameters $|D| = 0.0286\ \mathrm{cm}^{-1}$, $|E| = 0.0044\ \mathrm{cm}^{-1}$ was detected. When the primary acceptor was singly reduced either chemically or photochemically, a triplet state of a different spectral shape was observed, bearing the same D and E values and characteristic spin polarization pattern arising from RC radical pair recombination. The latter triplet state was strongly temperature dependent disappearing at $T = 100$ K, and had a much faster decay than the former one. Based on its properties, this triplet state was also ascribed to the photosystem II reaction center. A sequence of electron-transfer events in the reaction centers is proposed that explains the dependence of the triplet state properties on the reduction state of the iron–quinone primary acceptor complex.

*Keywords:* Photosystem II; Triplet state; Time-resolved EPR

## 1. Introduction

The reaction centers (RCs) of Photosystem II (PS II) of higher plants and cyanobacteria to a large degree resemble that of the purple photosynthetic bacteria (bRCs) [1]. Recently obtained X-ray structures of the PS II RCs from thermophilic cyanobacteria seem to support this view as concerns the spatial organisation of the electron-transfer cofactors [2–4]. In this context, it appeared attractive to assume a similarity of the photoinduced electron-transfer events in the two types of RCs. Thus, the concept dominant for a long time was that after excitation of the PS II RC, an electron from the primary donor chlorophyll (Chl) molecule P is transferred to the intermediary acceptor, pheophytin (Pheo), and further to the primary one-electron quinone acceptor $Q_A$ and to the secondary two-electron acceptor quinone $Q_B$, which have an iron ion, $Fe^{2+}$, in their vicinity [1,5]. This direct analogy with the bacterial primary processes, however, often meets with contradicting experimental evidence. Here, we will report on the study of the PS II RC triplet state. Such studies have played an important role in unveiling the mechanism of the primary processes in bacterial RCs [6]. In bRCs, the triplet state $^3P$ is populated in the course of recombination of the primary radical pair $P^+BPheo^-$ when further electron transfer is blocked by prereduction or removal of the primary quinone acceptor $Q_A$. In the bacterial case, P denotes the primary donor molecule, a bacteriochlorophyll (BChl) dimer, and BPheo, a bacteriopheophytin located in the bRC similar to the position of Pheo in the PS II RCs. The $^3P$ state in bRCs is easily detectable with cw EPR. Surprisingly, cw EPR detection of the corresponding state in PS II RCs appeared possible only in the case when the $Q_A$ molecule accepted two electrons under strong reducing conditions [7], or when it was absent altogether [8]. No EPR triplet signal could be observed when $Q_A$ was singly reduced. Still, using optical time-resolved spectroscopy it was possible to detect what was thought to be the triplet state $^3P$ also under the

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* Corresponding author. Tel.: +31 71 5275979; fax: +31 71 5275819.
*E-mail address:* Gast@Physics.LeidenUniv.nl (P. Gast).

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conditions of singly reduced $Q_A$ [9,10]. The triplet appeared to decay two or three orders of magnitude faster than the one in RCs with doubly reduced $Q_A$. In the present communication, we report on the first observation of the triplet state in PS II RCs with singly reduced primary quinone acceptor using time-resolved cw EPR (TREPR). The triplet state EPR properties are discussed in relation to the primary photochemical events in the RCs of PS II.

## 2. Materials and methods

Oxygen-evolving PS II core particles were routinely prepared from spinach according to Ref. [11] in BTS400 buffer, containing 20 mM Bis-Tris (pH 6.5), 20 mM $MgCl_2$, 5 mM $CaCl_2$, 10 mM $MgSO_4$, 0.4 M sucrose and 0.03% n-dodecyl-β-D-maltoside. $O_2$-evolving activity of the particles was 1200 μmol (mg Chl)$^{-1}$h$^{-1}$. After elution from the column, the core particles were concentrated under $N_2$ pressure in an Amicon vessel by using a 100-kDa filter and stored in liquid $N_2$ for later use at a concentration of 2 mg Chl/ml. Single reduction of $Q_A$ was achieved either by chemical reduction [7] or by photoreduction of non-treated samples at 260 K in the EPR cavity [12]. Non-treated samples were thawed and bubbled with argon for at least 5 min to decrease the $O_2$ content. To prepare EPR samples, buffer containing 100 mM MES (pH 6.5), 20 mM $MgCl_2$, 12.5 mM $MgSO_4$, 10 mM $CaCl_2$, 0.03% n-dodecyl-β-D-maltoside and 80% glycerol was bubbled with argon and added to the core particles under $N_2$ atmosphere in a glove box in the dark; the final glycerol concentration was 60% v/v. The samples were frozen in liquid $N_2$ in 4 mm I.D. quartz EPR tubes. To prepare chemically reduced samples, in the above procedure sodium dithionite and benzyl viologen were added to the MES buffer before mixing it with the core preparation, so that the final concentrations were 4 mM of dithionite (for single $Q_A$ reduction) or 8 mM dithionite and 100 μM benzyl viologen (for double $Q_A$ reduction). 200 mM sodium formate was also present for $g=1.82$ signal enhancement [7]. Incubation in the dark for single $Q_A$ reduction was conducted at room temperature for <5 min. For double reduction of $Q_A$, a series of incubation times ranging from 5 min to 240 min was tested. Throughout this study the samples had a chlorophyll concentration of 0.5 mg/ml. The reduction state of the primary acceptor was checked with cw EPR by measuring the $g=1.82$ $Fe^{2+}Q_A^-$ signal in the dark ([13,14], reviewed in [15]) and by measuring the RC triplet state EPR signal using a light-modulated (37 Hz) 300 W Xenon arc lamp in combination with second lock-in detection.

Fig. 1A shows the cw EPR signal of $Fe^{2+}Q_A^-$ in a sample prepared by photoreduction, and Fig. 1B shows the corresponding triplet state signal. Samples prepared by chemical single reduction of $Q_A$ demonstrated similar signals (data not shown). These properties are in agreement with earlier data [7] reported for singly reduced preparations. The weak steady-state triplet signal has zero-field splitting (ZFS) parameters corresponding to those of the PS II triplet [16]. We attribute the weak triplet signal (ca. 8% of the signal in doubly reduced preparations, see below) to PS II RCs that lost $Q_A$ during isolation and reduction procedures, or to a small fraction of doubly reduced $Q_A$.

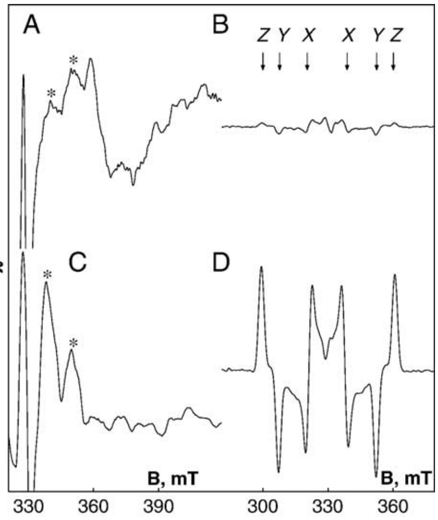

Fig. 1. cw EPR spectra of spinach Photosystem II core particles with singly (A, B) and doubly (C, D) reduced quinone acceptor $Q_A$. EPR conditions: microwave power 32 mW, modulation amplitude 1.6 mT, $T=5$ K (A, C); microwave power 0.1 mW, modulation amplitude 3.2 mT, $T=5$ K (B, D). In spectra (B, D) a 300-W Xenon lamp modulated at 37 Hz was used.

The low-temperature $Fe^{2+}Q_A^-$ signal could still be observed in doubly reduced PSII core samples after 5 min incubation under reducing conditions and declined with longer incubation times, as double reduction produces a non-paramagnetic state of the iron–quinone complex. No $Fe^{2+}Q_A^-$ signal could be detected after 40 min incubation, indicating complete double reduction of $Q_A$ (Fig. 1C). This is much shorter than reported by van Mieghem et al. [7], who obtained complete disappearance of the $Fe^{2+}Q_A^-$ signal in BBY preparations only after at least 4 h of incubation. This difference can be explained by an easier accessibility of the reductant to the redox components in spinach core complexes than in the larger BBY particles. Asterisks in Fig. 1A and C mark two high-field components of the $Mn^{2+}$ EPR signal appearing as the result of the well-known $Mn^{2+}$ release under reducing conditions (reviewed in [17]) and in the presence of formate [18]. In accordance with Ref. [7], double reduction also resulted in a strong triplet signal (Fig. 1D) with ZFS parameters characteristic of the PS II RC triplet state. The triplet signal had a maximum amplitude after 40 min incubation and declined with increasing incubation times. The triplet spin-polarization pattern (AEEAAE, where A stands for enhanced absorption, and E for emission of microwaves) indicates its $S-T_0$ population mechanism [19], produced by recombination of the reaction center primary radical pair (RP) [20].

Time-resolved EPR measurements were conducted as described earlier [21]. Samples were excited with 4-ns frequency-doubled Nd-YAG laser flashes at 532 nm with a repetition rate of 9.7 Hz. The flash intensity was attenuated to 0.3 mJ·cm$^{-2}$ to minimize accumulation of low-temperature photochemistry products. The EPR signal from the microwave mixer after wideband amplification was fed into a boxcar integrator. The signal sampling gate was set to a delay after flash (DAF) of 0.2 μs and duration of 1.3 μs. EPR spectra were accumulated by averaging the boxcar output while repeatedly sweeping the magnetic field. To obtain a triplet spectrum, $3-5\times10^5$ laser shots were applied. Signal decay curves were recorded by accumulating signals at field positions fixed at the X-peak maxima of the triplet spectra using a LeCroy digital oscilloscope. The sample temperature was controlled by an Oxford helium flow cryostat.

## 3. Results

Fig. 2 shows the TREPR triplet state spectra of doubly reduced core complexes. The spectrum in Fig. 2A was recorded at 10 K. Its shape is well simulated within the assumption of a fully $T_0$-populated, isotropically excited triplet with ZFS parameters $D=0.0286$ cm$^{-1}$, $E=0.0044$ cm$^{-1}$ (Fig. 2B). These parameters and the polarization pattern of AEEAAE correspond well to the triplet state attributed to the PS II reaction center [16,22]. It should be noted that the amplitude of the signal gradually decreased in the course of averaging. This was most likely due to photoaccumulation of reduced Pheo [14] due to electron donation to the oxidized primary donor from chlorophyll or carotenoid molecules of the RC [23], which converts RCs into a photochemically inactive state. At increased temperatures significantly smaller triplet state signals were noted. The triplet spectrum, however, remained detectable at 100 K (Fig. 2C).

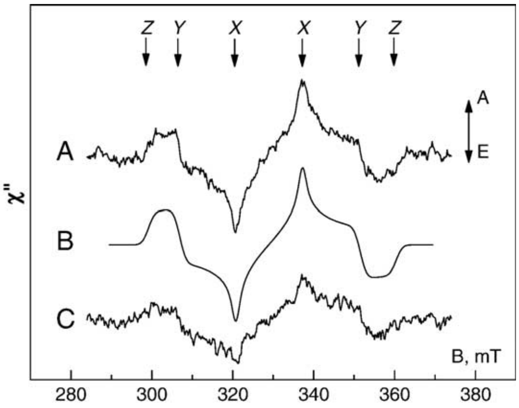

Fig. 2. TREPR spectra of PS II core particles with doubly reduced $Fe^{2+}Q_A$ acceptor complex. (A) spectrum recorded at $T=10$ K; (B) calculated $S-T_0$ polarized triplet state spectrum with ZFS parameters $D=+0.0286$ cm$^{-1}$, $E=+0.0044$ cm$^{-1}$; (C) spectrum recorded at $T=100$ K. Arrows at the right indicate the directions of absorption and emission of microwave power, denoted A and E, respectively. Arrows at the top show the positions of the spectrum canonical fields.

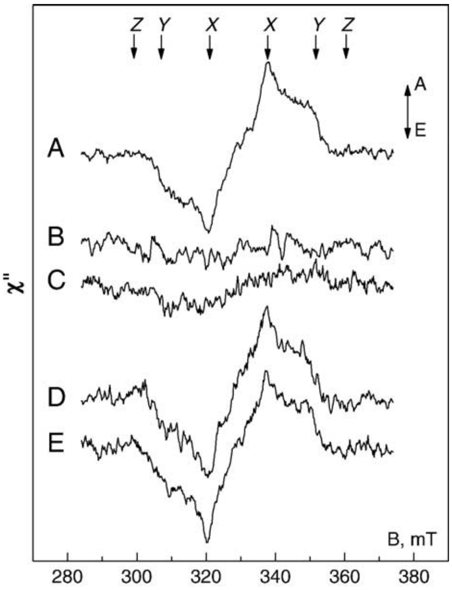

Fig. 3. TREPR spectra of PS II core particles with singly reduced $Fe^{2+}Q_A$ acceptor complex obtained by photoreduction at 260 K. (A) $T=10$ K; (B) $T=100$ K; (C) spectrum obtained with the same sample prior its photoreduction, $T=10$ K; (D) $T=6$ K; (E) $T=50$ K. Spectra A, B and C were scaled to Fig. 2; spectra D and E were normalized to the same amplitude as spectrum A.

With the high time resolution of the TREPR technique [20], it was possible to detect a short-lived triplet state in singly reduced PS II core particles. Fig. 3A shows the spectrum of this triplet state at 10 K recorded in a sample with photoreduced iron–quinone complex. The shape of the spectrum did not change much in the temperature range from 6 to 50 K (Fig. 3D, E). Chemically reduced samples gave an almost identical spectrum (data not shown), though with a slightly increased amplitude of the Z-components. The latter effect is probably due to double reduction of $Fe^{2+}Q_A^-$ in a fraction of the RCs and an admixture of the spectrum as in Fig. 2A. In contrast to doubly reduced samples, in singly reduced preparations an increase of temperature to 100 K led to an almost complete disappearance of the EPR signal (Fig. 3B). Such low triplet yield at elevated temperatures has been observed earlier in optical studies of the triplet state in singly reduced core preparations [24,25]. At low temperature, the EPR signal (Fig. 3A) decayed much faster than the signal of doubly reduced RCs (see below). Such behavior is also in agreement with the properties established optically [9]. We thus conclude that the EPR spectrum shown in Fig. 3A belongs to the triplet state detected earlier optically in singly reduced core particles [9,10]. The spectrum of Fig. 3A has ZFS parameters that are indistinguishable from the triplet state in doubly reduced samples (Fig. 2A). Its shape, however, is markedly different, with an almost complete absence of the Z-components. The (A)EEAA(E) spin polarization pattern, characteristic of the reaction center radical pair recombination triplet is, however, preserved.

The amplitude of the triplet spectrum in Fig. 3A is larger than of that in Fig. 2A, and no noticeable reduction of the amplitude during averaging was observed. This can be explained by a shorter lifetime of the precursor radical pair in RCs with singly reduced $Q_A$ [9], which decreases the probability of electron donation to the oxidized species of the RP, and thus slows down the process of reduced Pheo accumulation. We conclude that, in accordance with previous results [9,10,25], the yield of the triplet state in PS II RCs with singly reduced $Q_A$ does not differ much from that in doubly reduced samples. Prior to photoreduction of the sample, a weak signal of unspecified origin was observed (Fig. 3C), which may partially be ascribed to RCs that lack $Q_A$. The study of the dynamic properties of the spin-polarized signals with TREPR is not straightforward because of the strong influence of microwave-induced relaxation [26]. To obtain the intrinsic kinetic parameters, a series of signal decay measurements at progressively attenuated microwave power has to be performed. Ideally, a power level must be reached at which induced relaxation becomes much slower than the intrinsic decay processes. This condition, however, often cannot be reached due to the drop in EPR sensitivity at low microwave power. The lifetime of the triplet state in doubly reduced core particles at 10 K should be circa 1 ms [9,25]. In the present work, the signal decay (measured at the X-peaks, Fig. 2A) was determined by induced relaxation down to the lowest microwave power used (about 0.06 mW). At this power level and $T=10$ K its decay time was $\tau_e=30\pm6$ μs. Since the decay rate was still dependent on microwave power, the intrinsic decay rate is likely much slower, i.e., 1 ms. In contrast, the lifetime of the triplet signal in singly reduced preparations was practically independent of microwave power levels below 6 mW, with a decay time of $\tau_e=5\pm1$ μs at 10 K. Since the decay rate was not dependent on microwave power, this value represents the intrinsic dynamics of the triplet state. It coincides with the faster component of the triplet decay times measured optically [25] (at 20 K, a 50:50 ratio of components decaying with $t_{1/2}=2$ μs and 20 μs [9]). At 6 mW, we studied the temperature dependence of the triplet kinetics in singly reduced core particles. In Fig. 4, the amplitude of the kinetic traces measured at X-peaks is plotted vs. temperature. With the exception of the increase between 10 and 30 K, the temperature dependence is very similar to that measured earlier with time-resolved optical spectroscopy [25]. The non-monotonous behavior of the EPR-detected triplet signal in PS II RCs at 10–30 K like that in Fig. 4 has been observed before [21] and so far has no plausible explanation. The decay time of the signal decreases from 5 μs at $T=10$ K to $\tau_e=1.1\pm0.4$ μs at 70 K, the highest temperature used. The inset in Fig. 4 shows the kinetic trace at T=60 K and its mono-exponential fitting ($\tau_e=2.6\pm0.4$ μs). In the whole temperature range the kinetics remain within the time resolution of the spectrometer and are in a near-quantitative agreement with the lifetime of the fast triplet component obtained with time-resolved optical spectroscopy [25].

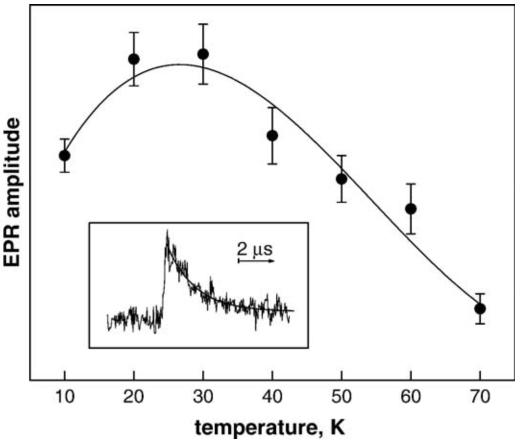

Fig. 4. Temperature dependence of the amplitude of the triplet state EPR signal decay kinetics measured at the X canonical points of the spectrum as in Fig. 3A (singly photoreduced $Fe^{2+}Q_A$ acceptor complex). Inset shows the kinetic trace at $T=60$ K and the result of its mono-exponential fit.

## 4. Discussion

There are several properties of the triplet state in singly reduced PS II core preparations that demand explanation: (1) its spectral shape; (2) the strong temperature dependence of its amplitude; (3) rapid acceleration of its decay as temperature is increased. We will address these issues assuming that this triplet state, like the triplet state in RCs with doubly reduced $Q_A$ [27,28], at low temperature is localized on a monomeric chlorophyll molecule, presumably $B_A$ in the active branch of the RC. We will also rely upon the structural [2–4] and optical [29,30] data obtained with PS II RCs of cyanobacteria.

### 4.1. EPR spectral shape of the triplet state signal

The decrease in intensity of the Z-peaks of the low-temperature EPR spectrum recorded in singly reduced core preparations (Fig. 3A) may have several reasons:

- superposition of signals from triplet states of the core light-harvesting antenna subunits CP43 and CP47. The signals from monomeric Chls have similar ZFS parameters and are known to possess spin polarization pattern EEEAAA [31]. An admixture of signals from such triplet states would decrease the observed intensity of the Z-peaks. However, the contribution from these antenna Chls will be largely temperature independent whereas the spectral intensity of the RC triplet state decreases with temperature (Fig. 3B). This would lead to a change in the overall spectral shape, which is not the case and thus supports the notion that no contribution from antenna Chls is present. The effect should also be pronounced in the spectrum of the doubly reduced state (Fig. 2A), which is not the case. In addition, no signals of sufficient intensity were observed in non-treated preparations (Fig. 3C);

- anisotropic relaxation or deactivation of the triplet state, so that Z-components of its signal disappear from the spectrum faster than the Y- and X-components. To check this possibility, we measured a series of spectra at different DAF between 50 ns and 3 μs (data not shown). There was no visible change of the spectral shape, so this mechanism is unlikely;
- population of the triplet state as a result of spin evolution in a three-spin system comprised of the RP and the electron spin of the $Fe^{2+}Q_A^-$ acceptor. It has been shown that in such systems the population of triplet spin sublevels may deviate from pure $T_0$ [32–34], leading to changes in the EPR spectral shape. In RCs from the phototrophic bacterium *Blastochloris (B.) viridis* (previously *Rhodopseudomonas viridis*), it was observed that the triplet actually had lower than expected intensity of Z- and X-peaks [35–37]. It has been argued that the effect is well pronounced in *B. viridis* because of a strong magnetic interaction between $BPheo^-$, partner of the RP, and the $Fe^{2+}Q_A^-$ complex, which is manifested by the low-temperature doublet EPR signal. Splitting between the two lines (ca. 6 mT [13,38]) is a direct measure of the interaction. Noteworthy, in PS II RCs a doublet signal with a similar splitting has been recorded too (5.6 mT [39]), and was also attributed to $Pheo^-$ interacting with the $Fe^{2+}Q_A^-$ complex. There is, however, an important difference between the temperature dependence of the triplet state EPR signals in bacterial RCs and PS II RCs. The shape of the $^3P$ spectrum in *B. viridis* RCs experiences profound changes when the temperature is increased above 20 K [35–37], resulting from rapid acceleration of spin relaxation of the $Fe^{2+}Q_A^-$ acceptor [40]. As spin relaxation of the PS II acceptor $Fe^{2+}Q_A^-$ is also very temperature-sensitive (leading to disappearance of its signal above liquid helium temperatures [41]), one should expect a similar temperature effect on the PS II triplet state. However, no significant changes in the spectral shape of the triplet in singly reduced core particles could be observed in the temperature range from 6 to 50 K (Fig. 3D, E). We thus reject this mechanism as the major cause of the triplet state EPR spectral shape;
- anisotropy of the triplet yield. The yield of a triplet state populated by RP recombination depends on the interplay of three dynamic factors—the rate of singlet–triplet conversion of the RP, and its two recombination rates into the triplet and singlet states [34,42]. Anisotropy of the triplet yield can arise from anisotropy of the singlet–triplet conversion rate of the RP. This rate is determined by the difference of *g*-factors of the RP partners ($\Delta g$), hyperfine interactions between the unpaired electrons in the RP and nuclei (hfi), and magnetic dipolar and exchange interactions in the RP. In an external magnetic field, all these entities may be anisotropic, resulting in an $S-T_0$ mixing frequency that depends on the RP orientation relative to the magnetic field. If the RP recombines, producing a triplet or singlet state of one of its partners, at a rate comparable to the rate of spin evolution, the yields of triplet and singlet products of recombination may become anisotropic. Thus, to observe triplet yield anisotropy, the precursor radical pairs should be comparatively short-lived. Effects of anisotropic triplet yield have been observed at D-band (130 GHz) for the $^3P$ yield in bacterial RCs [43] leading to a distortion of its EPR line shape, and has been employed to explain the triplet state D-band EPR line shape for PS II RCs [44]. At D-band, the *g*-factor anisotropy is most important, as in the D-band magnetic fields (ca. 4.5 T) the $\Delta g$ contribution to the S–T conversion rate dominates. At X-band, however, the $\Delta g$ contribution is more than 10-fold less, and anisotropy will be determined by the interplay of dipolar, exchange and hyperfine interactions in the RP. The proposed triplet yield anisotropy will result in a less than 100% overall triplet yield. Preliminary calculations of the triplet spectrum in Fig. 3A show that the observed shape can indeed be achieved assuming yield anisotropy, which leads to a 75% overall triplet yield.

Radical pair decay in spinach photosystem II particles with singly reduced quinone acceptor at 20 K was found to be biphasic with a lifetime of the fast component $\tau_{1/2}$ of ca. 30 ns [9]. This RP lifetime is similar in bacterial RCs with singly reduced acceptor $Q_A$ (20–30 ns for *Rb. sphaeroides* in the temperature range 5–90 K, as reviewed in [45]). No apparent anisotropy of the primary donor triplet yield has been detected by EPR at X-band [37] in this case, because the RP lifetime is long enough for complete S–T spin evolution. It then would be surprising how anisotropy could appear in PS II, where the properties of the radical pair $P^+Pheo^-$ are quite similar to the bacterial RPs.

In [29], the population of a state $P^+B_A^-$ was suggested to be the first step of electron transfer in PS II. Noteworthy, generation of a similar state upon direct excitation of the monomeric BChl has been proposed for bacterial RCs and demonstrated on several mutant reaction centers (reviewed in [46]). It should be pointed out that in the pair $P^+B_A^-$, which is created in a singlet state from $^1B_A^*$ [29,30], no singlet–triplet mixing can develop: the two radicals of the RP are located close together, and their exchange interaction is quite strong (estimated to be 4.8 T [44]). Thus, spin evolution will only develop at the next electron transfer step, i.e., in the RP $P^+Pheo^-$. The two RPs are expected to have similar optical spectra. Actually, optical changes accompanying pheophytin and chlorophyll electrolytic reduction in solution in the $Q_y$ region are quite similar [47]. Further, in spinach core preparations the molecule of Pheo absorbs at about 685 nm (this value obtained at 1.7 K [12]), very close to the proposed 684 nm absorption of $B_A$ (at 5 K [29]). If so, the RP lifetime measured in the low-temperature optical studies [9,10] is the combined lifetime of the RPs $P^+B^-$ and $P^+Pheo^-$. The period of singlet–triplet conversion may be much shorter than this combined lifetime, depending on the period that RCs spend in the $P^+Pheo^-$ state. Population of the $P^+B^-$ and $P^+Pheo^-$ states depends on the relative energies of these RPs, and can be modulated by the charge state of the primary quinone acceptor. Since $Pheo^-$ is located nearer to $Q_A$, its energy will experience larger increase due to electrostatic repulsion with the singly reduced quinone, than the molecule of $B_A^-$. Thus, single reduction of quinone will shift the energy level of $P^+Pheo^-$ upward stronger, than the level of the $P^+B^-$ RP. For normal electron transfer, the energy level of $P^+Pheo^-$ in functional RCs should be lower than that of $P^+B^-$. Single reduction of quinone will bring the two levels closer together, increasing the lifetime of the $P^+B^-$ state, decreasing the time of spin evolution in the $P^+Pheo^-$ RP, and creating conditions for the triplet yield anisotropy. Such effect is absent in RCs with doubly reduced $Q_A$, as the reduction procedure results in electrically neutral $QH_2$ state [7].

### 4.2. Strong dependence of the triplet yield on temperature

Amplitudes of the kinetic curves measured on singly reduced samples (Fig. 4) decrease with increasing temperature, indicating that the triplet state yield decreases. A similar dependence was observed earlier optically [25]. As the RC triplet is populated via recombination of the precursor radical pair, the drop of the triplet yield means that other channels than the triplet channel of RP decay become more efficient. One of such channels is singlet RP recombination followed by energy transfer back to the light-harvesting antenna. The RP state in PS II is generally considered as a shallow excitation trap [30]. A negative charge located on the $Q_A$ molecule in singly reduced RCs will increase the energy of the RPs $P^+Pheo^-$ and $P^+B_A^-$, making the trap of excitation even more shallow. This will further shift the equilibrium towards the antenna, decreasing the yield of the triplet state. In RCs, in their functional physiological state (non-reduced $Q_A$), this effect will be absent, and forward electron transfer will proceed unhampered. Additionally, since the energy levels of $P^+Pheo^-$ and $P^+B_A^-$ come closer, the RP population will shift towards $P^+B_A^-$, resulting in less spin evolution and consequently lower triplet yield.

### 4.3. Rapid acceleration of the triplet state decay with temperature

This has been tentatively explained by thermoactivated T–T energy transfer from $^3B_A$ to the molecule of Pheo, followed by reversible electron transfer between $^3$Pheo and singly reduced $Q_A$ [22,25]. The data reported here enable us neither to confirm nor to reject this assumption, so for the time being, the proposed mechanism remains the only one that qualitatively explains the temperature dependence of the triplet state lifetime. It should be noted that using FDMR, a short-lived triplet state with slightly different ZFS parameters was observed earlier in thylakoids [48]. Additional work is needed to correlate this triplet state to the one reported here.

The difference of the triplet state properties in the PS II RCs with singly and doubly reduced primary quinone acceptor may be physiologically relevant, as discussed in [49]. Photo-damage may occur because $^3$Chl can transfer energy to triplet $O_2$ with formation of potentially damaging singlet-excited oxygen. In some organisms, singlet $O_2$ formation via this channel is suppressed by fast T–T transfer from triplet (bacterio)chlorophyll to a carotenoid molecule with subsequent rapid dissipation of excitation energy. PS II RCs also incorporate carotenoids. These molecules, however, are located too far from the triplet-bearing $B_A$ (ca. 30 Å, [4]) to play a role in efficient triplet state quenching [50]. Thus, when populated, $^3B_A$ may transfer its energy to $O_2$ and cause destructive effects on the PS II RC, especially the D1 subunit since $B_A$ is located here, and singlet $O_2$ will be generated in its direct vicinity. Reaction centers with singly and doubly reduced quinone acceptor may appear when the quinone pool between PS II and PS I of a plant is reduced. In the case of singly reduced $Q_A$, the populated triplet states are short-lived and also have a very low yield at 100 K. If we assume equally low triplet yields at environmental temperature, generation of singlet $O_2$ will not occur via this mechanism. This is beneficial to the organism since single $Q_A$ reduction can be overcome [51] if the plastoquinone pool is (partly) oxidized again resulting in restored electron transfer from $Q_A^-$ to $Q_B$ and degradation of a useful protein is prevented. On the other hand, the double quinone reduction is irreversible and leads to non-functional RCs. The long-lived triplet state in such RCs is able to produce singlet oxygen, which subsequently will destroy these non-active RCs, preferably the D1 subunit due to the localization of the triplet mostly on $B_A$, and stimulate re-synthesis of active reaction centers. Noteworthy, high intensity illumination under physiological conditions induces fast turnover of the D1 protein with an approximate period of 60 min, whereas the other PSII proteins including D2 undergo turnover with much slower rates (reviewed in [52,53]).

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